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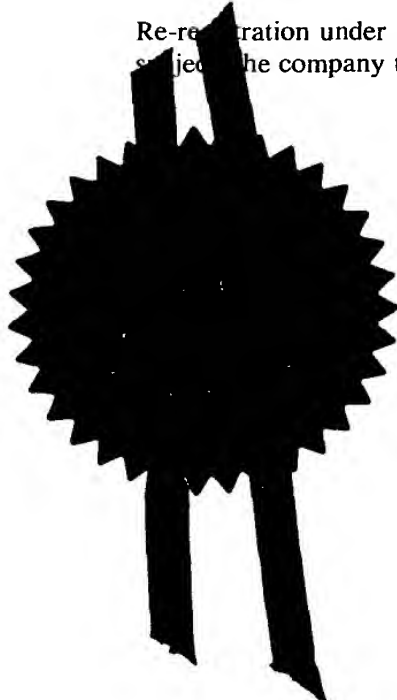
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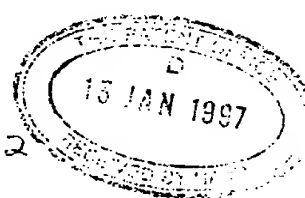
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2. Patent application number (The Patent Office will fill in this part)	13 JAN 1997	9700574.8	
3. Full name, address and postcode of the or of each applicant (underline all surnames)	CANCER RESEARCH CAMPAIGN TECHNOLOGY LIMITED CAMBRIDGE HOUSE 6-10 CAMBRIDGE TERRACE REGENT'S PARK LONDON NW1 4JL UNITED KINGDOM		
Patents ADP number (if you know it)	398450002		
If the applicant is a corporate body, give the country/state of its incorporation	UNITED KINGDOM		
4. Title of the invention	METHODS AND MEANS RELATING TO RETROTRANSPONON AND RETROVIRAL INTEGRATION		
5. Name of your agent (if you have one)	MEWBURN ELLIS		
"Address for service" in the United Kingdom to which all correspondence should be sent (including the postcode)	YORK HOUSE 23 KINGSWAY LONDON WC2B 6HP		
Patents ADP number (if you know it)	109006		
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8. Is a statement of inventorship and of right to grant of a patent required in support of this request? (Answer 'Yes' if: a) any applicant named in part 3 is not an inventor, or b) there is an inventor who is not named as an applicant, or c) any named applicant is a corporate body. See note (d))	YES		



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Description 52

Claim(s) 0

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11. I/We request the grant of a patent on the basis of this application.

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METHODS AND MEANS RELATING TO
RETROTRANSPOSON AND RETROVIRAL INTEGRATION

The present invention relates to the processes whereby retroviruses and retrotransposons (retroposons) insert their genetic material into the genome of a eukaryotic host cell in order to carry out a productive infection cycle. More specifically, it relates to proteins of the host cell that have now been found to be required for efficient retrotransposition, which are highly conserved throughout the eukaryotic kingdom but which are not required for cell functioning under most normal conditions. These proteins represent novel targets for anti-retroviral drugs. In addition, assay systems are provided with which anti-retroviral drugs can be screened and tested *in vivo* and *in vitro*.

The invention is based on the surprising discovery, contrary to prior teachings, that Ku-associated DNA repair mechanisms are involved in retrovirus and retrotransposon nucleic acid integration. Retrovirus and/or retrotransposon activity is shown by experimental work described herein to be inhibited in both yeast and mammalian cells where Ku function in the cells is reduced.

Retroviruses and retrotransposons

Retroviruses are RNA viruses that must insert a DNA copy (cDNA) of their genome into the host chromosome in order to carry out a productive

infection. When integrated, the virus is termed a provirus (Varmus, 1988). Some eukaryotic transposable DNA elements are related to retroviruses in that they transpose via an RNA intermediate. These elements, termed retrotransposons or retroposons, are transcribed into RNA, the RNA is copied into double-stranded (ds) DNA, then the dsDNA is inserted into the genome of the host cell.

The available evidence indicates that the integration of retroviruses and retrotransposons occurs through entirely analogous mechanisms, and that retroviruses can be viewed as retrotransposons with an extracellular phase of their life cycle. For example, the Ty1 and Ty5 retrotransposons of the yeast *Saccharomyces cerevisiae* have been shown to integrate into the host yeast genome by the same type of mechanism that is employed by mammalian retrotransposons and retroviruses to integrate into mammalian host cell DNA (Boeke et al., 1985; Garfinkel, 1985; Grandgenett and Mumm, 1990; Boeke and Sandmeyer, 1991).

Retroviruses are of considerable risk to human and animal health, as evidenced by the fact that retroviruses cause diseases such as acquired immune deficiency syndrome (AIDS; caused by human immunodeficiency virus; HIV-1), various animal cancers, and human adult T-cell leukaemia/lymphoma (Varmus, 1988). In many but not all cases, cancer formation by

certain retroviruses is a consequence of them carrying oncogenes. Furthermore, retroviral integration and retrotransposition can result in mutagenic inactivation of genes at their sites of insertion, or can result in aberrant expression of adjacent host genes, both of which can have deleterious consequences for the host organism. Retroviruses are also becoming used more and more commonly for gene delivery and are likely to play increasingly important roles in gene therapy. An understanding of how retroviruses function and how they can be controlled is therefore of great commercial and medical importance.

Over recent years, a vast amount of effort has been directed towards identifying inhibitors of retroviral infection because these agents have potential use in combatting retrovirally-borne diseases. To date, most drug development programmes have focused on virally-encoded products. However, given the short life cycle of retroviruses and their inherently high rates of genetic change, it is anticipated that a frequent problem with such strategies will be that drug resistant virus derivatives will arise through alterations of the virally-encoded target molecule (for example, Sandstrom and Folks, 1996 and references therein). Thus, most anti-retroviral drugs that interfere with virally-encoded proteins may only have a limited useful life-span. Another limitation of drugs that target

virus proteins is that many will not have a broad applicability and will be inherently highly specific to a particular virus or even a certain strain of a particular virus.

5

Retroviral integration

Given what is known about the retroviral life cycle, a further target for anti-retroviral therapeutics is to interfere with the integration of the viral cDNA into the host genome. Most importantly, this event is essential for efficient viral propagation (for example, Sakai et al., 1993; for reviews, see Varmus, 1988; Grandgenett and Mumm, 1990). In addition, since similar types of process are not believed to be essential for the functioning of most normally growing host cells, inhibitors of retroviral integration would not be expected to be particularly toxic to the host.

In light of these and other considerations, retroviral reverse transcriptases and integrases have been targeted for drug development. Although this has met with some success, high rates of genetic change by the targeted virus and variations between different viral strains is likely to limit the scope for anti-reverse transcriptase and anti-integrase drugs, particularly in the long term.

One way to surmount the problems outlined above

would be to identify host cell proteins that are required for efficient retroviral integration and derive drugs that inhibit these molecules. First, it would be very difficult or impossible for the virus to mutate in such a way that it could evade drug action. Second, such host cell proteins would be expected to be necessary for the propagation of most retroviruses, meaning that drugs that interfere with them would be effective against a wide spectrum of retrovirus types.

Until now, the idea of there being a host factor (or host factors) that is required for retroviral integration but is not necessary for normal host cell growth seemed unlikely. This is because several lines of research have indicated that all the steps needed for covalently linking retrovirus or retrotransposon cDNA to the target DNA molecule can be performed *in vitro* by purified retroviral integrase protein (for example, Craigie et al., 1990; Bushman et al., 1990; Katz et al., 1990; Grandgenett and Mumm, 1990). In addition, although host factors have been conceived to help with viral integration, it was assumed that these would correspond to "housekeeping proteins" that are essential for host cell viability. Thus, if host "helper" proteins did exist, it was expected that inhibiting them with drugs would not be worthwhile in a therapeutic context because this would also kill the cells of the host.

In spite of these predictions, the present

invention is surprisingly founded on the discovery that a series of host cell proteins are essential for efficient retrotransposon integration despite being unnecessary for host cell viability under most circumstances. These factors, which are components of a system termed the Ku-associated DNA repair apparatus, are therefore highly attractive targets for anti-retroviral therapy.

10 *The Ku-associated DNA repair system*

Previous work has revealed that the protein Ku is an essential component of the DNA repair apparatus in organisms ranging from humans, to *Drosophila melanogaster*, to *S. cerevisiae* (Jackson and Jeggo, 1995; Boulton and Jackson, 1996; Boulton and Jackson, 1996 and references therein). Specifically, the type of DNA repair process in which Ku is involved is termed illegitimate DNA end-joining or DNA double-strand break (DSB) repair. Since Ku binds to DNA DSBs *in vitro*, it has been proposed that Ku binds to DNA DSBs as they arise *in vivo* and helps to promote their efficient ligation. In addition, Ku is required for V(D)J recombination, a DNA "cut-and-paste" process that generates the antigen-binding molecules of the immune system of vertebrates (for reviews, see Lewis, 1994; Jackson and Jeggo, 1995).

In all organisms in which it has been identified, Ku exists as a heterodimer of two polypeptides of

approximately 70 kDa (termed Ku70 in humans; Yku70p or
HDF in *S. cerevisiae*) and 80 kDa (Ku80 or Ku86 in
humans; Yku80p in *S. cerevisiae*). References for this
are given below. Ku-defective cells are hypersensitive
5 to killing by ionising radiation or by radiomimetic
agents. However, in both yeast and mammalian systems,
lack of Ku function has minimal or undetectable effects
on cell viability and cell growth rates under normal
conditions (Boulton and Jackson, 1996a, Milne et al.,
10 Blunt et al.; Jackson and Jeggo and references therein;
Nussenzweig et al. and Zhu et al.).

Recently, additional components of the
Ku-associated DNA repair pathway have been identified.
One of these is the mammalian protein XRCC4,
15 deficiencies in which produce defects in DSB repair and
V(D)J recombination (Li et al., 1995). Others
identified are the yeast factors Rad50p, Mre11p, and
Xrs2p (S. Boulton JD and S.P. Jackson, unpublished data
and references given below) and the DNA ligase Lig4p
20 (S-H Teo and S.P. Jackson, unpublished data and
references given below). Since human cells possess
homologues of all these latter factors, they should
function in DSB repair and related processes in
essentially all eukaryotes.

25 Reeves and Stoecker, and Chan et al. disclose cDNA
and amino acid sequences of human Ku70. Cai et al.
disclose chromosomal location and expression of the
genes encoding Ku70 and Ku80 in human cell lines and

normal tissues, while Yaneva et al. disclose the cDNA-derived amino acid sequence of human Ku80. *Drosophila* Ku (Yolk Protein Factor 1) is disclosed by Jacoby and Wensink. Feldmann and Winnaker, Milne et al., Beall et al., and Boulton and Jackson give sequence and functional information about yeast (*Saccharomyces cerevisiae*) Ku70 and Ku80.

Alani et al. discloses the nucleic acid and amino acid sequences of yeast *RAD50*. Human *RAD50* and *MRE11* are described in Dolganov et al. and Petrini et al., respectively. Ivanov et al. describes *XRS2* of yeast.

Ku-associated DNA repair, which is an illegitimate DNA end repair mechanism, requires a discrete set of genes products (as discussed), and is a separate pathway from other DNA repair pathways which include homologous recombination repair, nucleotide excision repair, base excision repair and DNA mismatch repair.

Rationale for the experimental approach adopted in making the present invention

The present invention has been made by testing whether Ku plays a positive or negative role in the normal life cycle of retrotransposons, despite several lines of evidence suggesting that this is very unlikely to be the case.

One reason for the inventor's interest was that linear cDNA is generated as a intermediate during the

life cycle of retroviruses and retrotransposons,
raising the possibility that Ku may bind to it. The
prevailing view, however, is that Ku will be unable to
bind the viral cDNA, as this is assumed to be always
5 associated tightly with virally-encoded factors.
Nevertheless, since unpublished biochemical data from
the inventor's laboratory indicate that Ku is able to
bind to DNA with a tenacity almost unrivalled by any
other characterised protein, it was felt that Ku
10 gaining access to the retrotransposon cDNA could not be
ruled out.

The second rationale for the investigations was
the inventor's belief in a possibility that the host
Ku-associated DNA DSB repair apparatus may play a role
15 in retrovirus and retrotransposon integration.
Although the retrotransposon or retrovirus integrase
carries out all the steps needed to covalently link the
cDNA to the target DNA molecule (for example, Craigie
et al., 1990; Bushman et al., 1990; Katz et al., 1990),
20 "single-strand gap filling and ligation" steps are
needed before the retroviral/retrotransposon DNA can be
incorporated stably. Despite the fact that the
Ku-associated DNA repair apparatus is not known to
function in single-strand gap-filling and ligation
25 reactions, it is nevertheless demonstrated below that
it is indeed required for the efficient integration of
the yeast Ty1 and Ty5 retrotransposons and also effects
the integration of retroviral cDNA into mammalian

cells.

These surprising findings provide indication that the Ku-associated DNA repair apparatus plays a ubiquitous role in retrotransposon integration processes, and open up opportunities for
5 anti-retroviral action, as discussed.

According to one aspect of the present invention, there is provided a method of inhibiting retrovirus
10 and/or retrotransposon activity by means of a substance identified as an inhibitor of Ku-associated DNA repair. Methods of treatment of the human or animal body by way of therapy may be excluded.

However, a further aspect of the present invention provides the use of a substance identified as an
15 inhibitor of Ku-associated DNA repair in the manufacture of a medicament for inhibiting retrovirus and/or retrotransposon activity.

Another aspect of the present invention provides a
20 substance identified as an inhibitor of Ku-associated DNA repair for use in inhibiting retrovirus and/or retrotransposon activity.

A further aspect of the present invention provides the use of a substance identified as an inhibitor of
25 Ku-associated DNA repair in inhibiting retrovirus and/or retrotransposon activity.

The substance may be provided in a composition which includes at least one other component, for

instance a pharmaceutically acceptable excipient, as discussed further below.

5 The substance may be provided *in vivo* to cells in a human or animal body, by way of therapy (which may include prophylaxis), or *in planta*, *ex vivo* or *in vitro*. This too is discussed further elsewhere herein.

Integration of a retrovirus and/or retrotransposon into the genome of a cell may be inhibited by treatment of the cell with a substance which is an inhibitor of
10 Ku-associated DNA repair. Examples of such substances include wortmannin and LY294002.

Inhibition of Ku-associated DNA repair may be achieved in any of numerous different ways, without limitation to the nature and scope of the present
15 invention.

In certain embodiments of the present invention, Ku itself is targeted for inhibition, that is to say that Ku's involvement in Ku-associated DNA repair is inhibited in order to inhibit Ku-associated DNA repair.
20 Ku is only functional as a heterodimer (of subunits Ku70 and Ku80). One way, therefore, of inhibiting Ku activity is to inhibit interaction between the two subunits. Another way is to use a substance that inhibits interaction of Ku with DNA or another
25 component of the Ku-associated DNA repair pathway. Otherwise, Ku itself need not be targeted and the function one or more other components of the Ku-associated DNA repair pathway may be inhibited

(discussed further below). Of course, a substance may inhibit activity of a component of the pathway such as Ku not (or not solely) by inhibiting physical interaction between the component and another but by binding at an active site or by binding in a way that has a steric effect on the conformation of an active site and thus activity of the component. Precisely how the activity or function of a component of the pathway is inhibited need not be relevant to practising the present invention.

The sequences of various components of the Ku-associated DNA repair pathway in humans and yeast are available from the GenBank database, under the following accession numbers: human Ku70 - J04611; human Ku80 - M30938; *S. cerevisiae* Ku70 - X70379; *S. cerevisiae* Ku80 - Z49702; human ligase IV - X83441; *S. cerevisiae* ligase IV - YOR005c on the right arm of *S. cerevisiae* chromosome XV, accession number Z74913; human Rad50 - U63139; *S. cerevisiae* Rad50p - X14814; human Mre11 - U37359; *S. cerevisiae* Mre11 - D11463; human XRCC4 - U40622 (334 amino acid residue open reading frame); *S. cerevisiae* Xrs2p - L22856.

The activity or function of a component of the Ku-associated DNA repair pathway (such as Ku) may be inhibited, as noted, by means of a substance that interacts in some way with the component. An alternative employs regulation at the nucleic acid level to inhibit activity or function by down-

regulating production of the component.

For instance, under-expression of a gene may be achieved using anti-sense technology. The use of anti-sense genes or partial gene sequences to down-regulate gene expression is now well-established.

Antisense oligonucleotides may be designed to hybridise to the complementary sequence of nucleic acid, pre-mRNA or mature mRNA, interfering with the production of a component of the Ku-associated DNA repair pathway, such as Ku, or a subunit thereof, encoded by a given DNA sequence, so that its expression is reduced or completely or substantially completely prevented. In addition to targeting coding sequence, antisense techniques may be used to target control sequences of a gene, e.g. in the 5' flanking sequence, whereby the antisense oligonucleotides can interfere with expression control sequences. The construction of antisense sequences and their use is described for example in Peyman and Ulman, Chemical Reviews, 90:543-584, (1990) and Crooke, Ann. Rev. Pharmacol. Toxicol., 32:329-376, (1992).

Oligonucleotides may be generated *in vitro* or *ex vivo* for administration or anti-sense RNA may be generated *in vivo* within cells in which down-regulation is desired.

Thus, double-stranded DNA may be placed under the control of a promoter in a "reverse orientation" such that transcription of the anti-sense strand of the DNA

yields RNA which is complementary to normal mRNA transcribed from the sense strand of the target gene. The complementary anti-sense RNA sequence is thought then to bind with mRNA to form a duplex, inhibiting translation of the endogenous mRNA from the target gene into protein. Whether or not this is the actual mode of action is still uncertain. However, it is established fact that the technique works.

The complete sequence corresponding to the coding sequence in reverse orientation need not be used. For example fragments of sufficient length may be used. It is a routine matter for the person skilled in the art to screen fragments of various sizes and from various parts of the coding or flanking sequences of a gene to optimise the level of anti-sense inhibition. It may be advantageous to include the initiating methionine ATG codon, and perhaps one or more nucleotides upstream of the initiating codon. A suitable fragment may have about 14-23 nucleotides, e.g. about 15, 16 or 17.

Many known techniques and protocols for manipulation of nucleic acid, for example in preparation of nucleic acid constructs, mutagenesis, sequencing, introduction of DNA into cells and gene expression, and analysis of proteins, are described in detail in *Current Protocols in Molecular Biology*, Ausubel et al. eds., John Wiley & Sons, 1992, and *Molecular Cloning: a Laboratory Manual*: 2nd edition, Sambrook et al., 1989, Cold Spring Harbor Laboratory

Press.

It is well known that pharmaceutical research leading to the identification of a new drug may involve the screening of very large numbers of candidate substances, both before and even after a lead compound has been found. This is one factor which can make pharmaceutical research very expensive and time-consuming. Means for assisting in the screening process can have considerable commercial importance and utility. Such means for screening for substances potentially useful in inhibiting retroviral and/or retrotransposon activity is provided according to the present invention. Substances identified as modulators of Ku-associated DNA repair represent an advance in the fight against retroviral diseases (for instance), since they provide basis for design and investigation of therapeutics for *in vivo* use.

A method of screening for a substance which inhibits retrovirus and/or retrotransposon activity may include contacting one or more test substances with one or more components of the Ku-associated DNA repair pathway of an organism of interest in a suitable reaction medium, and testing for substance/component interaction, e.g. by assessing activity of the Ku-associated DNA repair pathway or component thereof and comparing that activity with the activity in comparable reaction medium untreated with the test substance or

substances. A difference in activity between the treated and untreated samples is indicative of a modulating effect of the relevant test substance or substances. It may be sufficient, at least as a preliminary, to assess mere physical interaction between test substance and pathway component or subunit thereof in test samples, rather than actual biochemical activity.

In further aspects the present invention relates to the screening of candidate substances for potential as inhibitors of retrovirus and/or retrotransposon activity. More particularly, it provides a method by which test substances can be screened for their ability to affect Ku-associated DNA repair. Test substances may be screened for inhibition or activation of the pathway, though clearly inhibitors of the pathway are of primary interest.

According to a further aspect of the present invention there is provided a method of screening for a substance which is an inhibitor of retrovirus and/or retrotransposon activity, particularly nucleic acid integration or transposition of retrovirus and/or retrotransposon, which includes:

- providing a Ku-associated DNA repair pathway;
- exposing the pathway to a test substance under conditions which would normally lead to the activation of the Ku-associated DNA repair pathway; and
- looking for an end-point indicative of activation

of the Ku-associated DNA repair pathway;

whereby inhibition of that end-point indicates inhibition of the Ku-associated DNA repair pathway by the test substance.

5 The pathway may be provided in a cell to be exposed to the test substance, or the assay may be performed on a *in vitro* Ku-associated DNA repair system that measures the accuracy and efficiency of joining together DNA strand breaks that have been created by
10 treating intact DNA with restriction endonucleases, chemicals, or radiation.

 Activation of the Ku-associated DNA repair pathway may be caused by DNA double-strand breaks (DSBs), single strand gaps in the DNA double helix and by other
15 disruptions to the DNA double-helix. These structures exist at the ends of retroviral and retrotransposon DNA and occur as intermediates in the retroviral integration and retrotransposition process. To assay for Ku-associated repair, retrovirus or retroviral DNA,
20 intermediates in retroviral integration or retrotransposon integration, or synthetic preparations of DNA that mimic any of these may be provided. The activation of the Ku-associated DNA repair pathway leads to the protection of DNA from excessive
25 degradation and results in perturbations in the DNA double-helix via the ligation of DNA DSBs or single-strand breaks.

 The end-point of the screen may be therefore the

repair of such perturbations. That a substance is inhibitory of the Ku-associated DNA repair pathway may be verified by hypersensitivity of mammalian cells to ionising radiation (e.g. Jackson and Jeggo, and refs
5 therein) or by rejoining of double-strand breaks (e.g. in a plasmid) *in vivo* (Boulton and Jackson, 1996a, 1996b). Biochemical methods, such as PCR or nucleic acid hybridisation/detection methods, may be used, e.g. to detect the chemical structure of integration
10 products. Retroviral integration and/or retrotransposition may be scored for example by detection using standard genetic, biochemical or histological techniques.

Prior to, as well as or instead of being screened
15 for ability actually to affect Ku-associated DNA repair activity, test substances may be screened for ability to interact with a component of the pathway (such as Ku or one or both of the subunits thereof) e.g. in a yeast two-hybrid system (which requires that both the
20 polypeptide component and the test substance can be expressed in yeast from encoding nucleic acid). This may for example be used as a coarse screen prior to testing a substance for actual ability to modulate activity.

25 Thus, in a further aspect, the present invention provides a method of screening for a substance which is an inhibitor of retrovirus and/or retrotransposon activity, particularly nucleic acid integration of

retrovirus and/or retrotransposon, which includes:

providing a component of a Ku-associated DNA repair pathway;

exposing the component to a test substance;

5 determining interaction between the component and the test substance.

A yeast two-hybrid system (e.g Evan et al. J.M. Mol. Cell. Biol. 5, 3610-3616 (1985); Fields & Song Nature 340, 245-246 (1989)) may be used to identify substances that interact with a Ku-associated DNA repair pathway component or subunit thereof. This system generally utilises a yeast containing a GAL4 responsive promoter linked to β -galactosidase gene and to a gene (His3) that allows the yeast to grow in the absence of the amino acid histidine and to grow in the presence of the toxic compound 3-aminotriazole. The pathway component or subunit may be cloned into a yeast vector that will express the protein as a fusion with the DNA binding domain of GAL4. The yeast may then be transformed with DNA libraries designed to express test polypeptides or peptides as GAL4 activator fusions. Yeast that have a blue colour on indicator plates (due to activation of β -galactosidase) and will grow in the absence of histidine (and the presence of 3-aminotriazole) may be selected and the library plasmid isolated. The library plasmid may encode a substance that can interact with the Ku-associated DNA repair pathway component or subunit thereof.

A variation on this may be used to screen for substances able to disrupt interaction between two components of the Ku-associated DNA repair pathway, or the subunits of a such a component (e.g. the Ku70 and Ku80 subunits of Ku - Ku is only functional as a heterodimer). For instance, the two components or subunits may be expressed in a yeast two-hybrid system (e.g. one as a GAL4 DNA binding domain fusion, the other as a GAL4 activator fusion) which is treated with test substances. The absence of the end-point which normally indicates interaction between the components or subunits (e.g. the absence of a blue colour in the exemplary system outlined above) when a test substance is applied indicates that that substance disrupts interaction between the two components or subunits, and may therefore inhibit Ku-associated DNA repair, indicative of potential as an inhibitor of retrovirus and/or retrotransposon activity.

For potential therapeutic purposes the Ku-associated DNA repair pathway or one or more components (or subunits) thereof used in the assay may be human, or mammalian or bird bearing in mind veterinary applications. However, given the ease of manipulation of yeast, and the good conservation between Ku-associated DNA repair components in different eukaryotes, an assay according to the present invention may involve applying test substances to a yeast system with the expectation that similar results will be

obtained using the substances in mammalian, e.g. human, systems. In otherwords, a substance identified as being able to inhibit Ku-associated DNA repair in yeast is likely to be able to inhibit Ku-associated DNA repair in other eukaryotes. A further approach, as discussed, is to use yeast cells expressing one or more components (e.g. Ku) or subunits (e.g. Ku70/Ku80) of the Ku-associated DNA repair pathway of another eukaryote, e.g. human. A plant Ku-associated repair pathway or one or more components thereof may be employed in an assay according to the present invention, to test for substance useful in inhibiting retroviral and/or retrotransposon activity in the plant or plants generally.

15

Following identification of a substance which modulates or affects Ku-associated DNA repair and/or interaction between components of the pathway or subunits thereof, the substance may be investigated further, in particular for its ability to inhibit retroviral and/or retrotransposon activity. Furthermore, it may be manufactured and/or used in preparation, i.e. manufacture or formulation, of a composition such as a medicament, pharmaceutical composition or drug. These may be administered to individuals.

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Thus, the present invention extends in various aspects not only to a substance identified as

inhibiting retroviral and/or retrotransposon activity in accordance with what is disclosed herein, but also a pharmaceutical composition, medicament, drug or other composition comprising such a substance, a method
5 comprising administration of such a composition to a patient, e.g. for treatment (which may include preventative treatment) of a retroviral disorder, use of such a substance in manufacture of a composition for administration, e.g. for treatment of a retroviral
10 disorder, and a method of making a composition comprising admixing such a substance with a pharmaceutically acceptable excipient, vehicle or carrier, and optionally other ingredients.

A substance that tests positive in an assay
15 according to the present invention or is otherwise found to inhibit retroviral and/or retrotransposon activity by inhibition of Ku-associated DNA repair may be peptide or non-peptide in nature. Non-peptide "small molecules" are often preferred for many *in vivo*
20 pharmaceutical uses. Accordingly, a mimetic or mimic of the substance (particularly if a peptide) may be designed for pharmaceutical use.

The designing of mimetics to a known
pharmaceutically active compound is a known approach to
25 the development of pharmaceuticals based on a "lead" compound. This might be desirable where the active compound is difficult or expensive to synthesise or where it is unsuitable for a particular method of

administration, e.g. peptides are unsuitable active agents for oral compositions as they tend to be quickly degraded by proteases in the alimentary canal. Mimetic design, synthesis and testing is generally used to
5 avoid randomly screening large number of molecules for a target property.

There are several steps commonly taken in the design of a mimetic from a compound having a given target property. Firstly, the particular parts of the
10 compound that are critical and/or important in determining the target property are determined. In the case of a peptide, this can be done by systematically varying the amino acid residues in the peptide, eg by substituting each residue in turn. Alanine scans of
15 peptide are commonly used to refine such peptide motifs. These parts or residues constituting the active region of the compound are known as its "pharmacophore".

Once the pharmacophore has been found, its
20 structure is modelled to according its physical properties, eg stereochemistry, bonding, size and/or charge, using data from a range of sources, eg spectroscopic techniques, X-ray diffraction data and NMR. Computational analysis, similarity mapping (which
25 models the charge and/or volume of a pharmacophore, rather than the bonding between atoms) and other techniques can be used in this modelling process.

In a variant of this approach, the three-

dimensional structure of the ligand and its binding partner are modelled. This can be especially useful where the ligand and/or binding partner change conformation on binding, allowing the model to take
5 account of this in the design of the mimetic.

A template molecule is then selected onto which chemical groups which mimic the pharmacophore can be grafted. The template molecule and the chemical groups grafted on to it can conveniently be selected so that
10 the mimetic is easy to synthesise, is likely to be pharmacologically acceptable, and does not degrade *in vivo*, while retaining the biological activity of the lead compound. Alternatively, where the mimetic is peptide based, further stability can be achieved by
15 cyclising the peptide, increasing its rigidity. The mimetic or mimetics found by this approach can then be screened to see whether they have the target property, or to what extent they exhibit it. Further optimisation or modification can then be carried out to
20 arrive at one or more final mimetics for *in vivo* or clinical testing.

A substance for inhibiting retrovirus and/or retrotransposon activity in accordance with any aspect of the present invention may be formulated in a
25 composition. A composition may include, in addition to said substance, a pharmaceutically acceptable excipient, carrier, buffer, stabiliser or one or more other materials well known to those skilled in the art.

Such materials should be non-toxic and should not interfere with the efficacy of the active ingredient. The precise nature of the carrier or other material may depend on the route of administration, e.g. oral,
5 intravenous, cutaneous or subcutaneous, nasal, intramuscular, intraperitoneal routes.

Pharmaceutical compositions for oral administration may be in tablet, capsule, powder or liquid form. A tablet may include a solid carrier such
10 as gelatin or an adjuvant. Liquid pharmaceutical compositions generally include a liquid carrier such as water, petroleum, animal or vegetable oils, mineral oil or synthetic oil. Physiological saline solution, dextrose or other saccharide solution or glycols such
15 as ethylene glycol, propylene glycol or polyethylene glycol may be included.

For intravenous, cutaneous or subcutaneous injection, or injection at a particular site of affliction, the active ingredient will be in the form
20 of a parenterally acceptable aqueous solution which is pyrogen-free and has suitable pH, isotonicity and stability. Those of relevant skill in the art are well able to prepare suitable solutions using, for example, isotonic vehicles such as Sodium Chloride Injection,
25 Ringer's Injection, Lactated Ringer's Injection. Preservatives, stabilisers, buffers, antioxidants and/or other additives may be included, as required.

Whether it is a polypeptide, peptide, nucleic acid

molecule, small molecule or other pharmaceutically useful compound according to the present invention that is to be given to an individual, administration is preferably in a "prophylactically effective amount" or
5 a "therapeutically effective amount" (as the case may be, although prophylaxis may be considered therapy), this being sufficient to show benefit to the individual. The actual amount administered, and rate and time-course of administration, will depend on the
10 nature and severity of what is being treated. Prescription of treatment, e.g. decisions on dosage etc, is within the responsibility of general practitioners and other medical doctors, and typically takes account of the disorder to be treated, the
15 condition of the individual patient, the site of delivery, the method of administration and other factors known to practitioners. Examples of the techniques and protocols mentioned above can be found in Remington's Pharmaceutical Sciences, 16th edition,
20 Osol, A. (ed), 1980.

Targeting therapies may be used to deliver the active agent more specifically to certain types of cell, by the use of targeting systems such as antibody or cell specific ligands. Targeting may be desirable
25 for a variety of reasons; for example if the agent is unacceptably toxic, or if it would otherwise require too high a dosage, or if it would not otherwise be able to enter the target cells.

Instead of administering these agents directly, they may be produced in the target cells by expression from an encoding gene introduced into the cells. The vector may be targeted to the specific cells to be
5 treated, or it may contain regulatory elements which are switched on more or less selectively by the target cells.

The agent may be administered in a precursor form, for conversion to the active form by an activating
10 agent produced in, or targeted to, the cells to be treated.

A composition may be administered alone or in combination with other treatments, either
15 simultaneously or sequentially dependent upon the condition to be treated.

The experimental basis for the invention and illustrative embodiments of the invention will now be described in more detail, with reference to the
20 accompanying drawings. All publications mentioned in the text are incorporated herein by reference.

Figure 1: Inactivation of *YKU70* leads to reduced frequencies of Ty1 transposition. Transposition
25 frequencies of Ty1 in wild-type *Saccharomyces cerevisiae* strain W303-1A (Wild-type), in a W303-1A derived *yku70* mutant yeast strain (*yku70*), or in the *yku70* mutant strain containing an episomal plasmid that

expresses the wild-type *YKU70* gene (*yku70/pYKU70*) were calculated relative to wild-type yeast (for details of yeast strains and *YKU70* plasmid, see Boulton and Jackson 1996). All transposition assays were performed as follows. Yeast strains were transformed with pGTy1-H3m*HIS3*AI (Curcio and Garfinkel, 1991) and plated to select for the *URA3* gene on this plasmid. The plasmid also contains a galactose-inducible Ty1 element which contains a *HIS3* gene with an artificial intron in the reverse orientation. Expression of the *HIS3* gene is dependent on transcription and transposition of the Ty1 element (Curcio and Garfinkel, 1991). Individual colonies were picked and grown in galactose-containing media overnight at 30°C. The optical density of cultures was measured at 595 nm to determine cell density, and plating on synthetic complete medium lacking uracil was used to determine cell viability. Cells were also plated onto synthetic complete medium containing glucose and lacking histidine, and the number of colonies arising divided by the total cell count was obtained as a measure of the Ty1 transposition frequency in each strain tested. Frequencies are shown relative to wild type, which had a mean transposition frequency of 1.5×10^{-4} .

Figure 2: Inactivation of *YKU80* but not *RAD52* or *SGS1* leads to reduced frequencies of Ty1 transposition. The wild-type strain W303-1A (Wild-type) or W303-1A derived strains deficient in *YKU80* (*yku80*), *RAD52*

(*rad52*) or *SGS1* (*sgs1*) were tested for their ability to support Ty1 retrotransposition as described in the legend to Fig. 1. Transposition frequencies are shown normalised to the wild-type strain.

5 Figure 3: Transposition by Ty5 is debilitated by mutations in *YKU70*. Yeast strains were transformed with a plasmid containing the Ty5 element under the control of the *GAL1* promoter, and containing the same *HIS3* artificial intron construct as pGTy1-H3mHIS3AI
10 (Zou et al. 1996). Assays were performed as described in Fig. 1.

 Figure 4: Ku does not appear to affect the generation of Ty1 VLPs. VLPs were purified from the wild-type strain W303-1A (Wild-type) or from a
15 derivative of this strain containing a disruption of *YKU70* (*yku70*; see Boulton and Jackson, 1996 for strain details). The protocol for purification of virus like particles (VLPs) was adapted from Eichinger and Boeke, 1988. Briefly, 5 ml of an overnight culture of yeast
20 cells was grown in synthetic complete (SC) yeast medium containing glucose but lacking uracil. Cells were then pelleted by centrifugation, washed with water and then used to inoculate 50 ml of SC containing galactose and lacking uracil. After growing for 24 h at 22 °C, cells
25 were harvested by centrifugation, washed with water and lysed by glass bead disruption in the presence of 1 ml of Buffer B/Mg²⁺ (10 mM HEPES pH 7.6, 15 mM KCl, 3 mM DTT, 10 µg/ml aprotinin, 5 mM MgCl₂). The lysate was

then centrifuged at 12,000 x g, and the supernatant was loaded onto a sucrose gradient (1 ml 70% sucrose in Buffer B without Mg^{2+} and with 10 mM EDTA, followed by 1 ml of this containing 30% sucrose, followed by 4 ml of this containing 20% sucrose). The sucrose gradients were centrifuged in an SW-40 rotor at 4°C for 3 h, then ten 0.7 ml fractions were collected and stored at 4°C. Each fraction was tested for reverse transcriptase activity as described previously by measuring the incorporation of [α - ^{32}P]dGTP into an oligo(dG)/poly(C) primer-template complex (Garfinkel et al. 1985). A representative profile of reverse transcriptase activity of various gradient fractions is presented. Positive fractions were pelleted at 50,000 x g overnight at 4°C, resuspended in 10 μ l of Buffer B/ Mg^{2+} , and were stored at 4°C for use in all subsequent assays.

Figure 5: VLPs isolated from Yku70p-deficient yeasts are competent for Ty1 integrase-mediated transposition *in vitro*. *In vitro* transposition was assayed using VLPs that had been purified from a wild-type or a yku70 mutant (yku70) yeast strain, as described in Fig. 4. Reactions contained 3 ml VLPs or Buffer B/ Mg^{2+} (negative control), 10 mM Tris pH 8.0, 15 mM $MgCl_2$, 1 mM DTT, 5 % polyethylene glycol, and 1 mg of supercoiled plasmid DNA (pRS416; Stratagene). Reactions were incubated for 30 min at 25°C, and stopped by the addition of proteinase K to 0.5 mg/ml

and SDS to 1.4 % followed by a further incubation at 25°C for 3h. Next, the DNA was extracted with phenol/chloroform, precipitated with ethanol, washed with 70 % ethanol, resuspended in 10 mM Tris pH 8.0, and transformed into XL-1 cells (Stratagene). Colony hybridization was performed using a random-primed probe corresponding to the *HIS3* gene. Positive colonies were counted relative to total colonies to give the average transposition frequency. Positive colonies were picked to ensure they contained the Ty1 element; as expected, reactions containing no VLPs yielded no positively hybridising colonies.

EXPERIMENTAL WORK

To test the potential involvement of Ku in retrotransposition, the yeast *S. cerevisiae* was used as a model system. Notably, recent studies have revealed that the Ku-associated DNA DSB repair system is highly conserved between yeast and humans (Boulton and Jackson, 1996; Boulton and Jackson, 1996 and references therein). Furthermore, it is clear that the yeast retrotransposons Ty1 and Ty5 transpose through mechanisms that are highly related to those of retrotransposons and retroviruses in other organisms, including humans (Boeke et al., 1985; Garfinkel, 1985; Boeke and Sandmeyer, 1991).

Yku70p is required for effective transposition by Ty1

To assess the efficiency of retrotransposition, the galactose-inducible Ty1 transposition system (Curcio and Garfinkel 1991) was initially utilised. In this system, a *his3* mutant derivative of the yeast strain under investigation is generated such that it contains an episomal replicating plasmid that directs the expression of Ty1 RNA from a galactose-inducible promoter. Importantly, the Ty1 element contains within it the *S. cerevisiae* *HIS3* gene that is interrupted by an intron in reverse (anti-sense) orientation. Thus, the yeast strain is phenotypically *his*⁻ and cannot grow in the absence of histidine in the growth medium. However, since the intron is in the sense orientation with respect to the Ty1 retroelement, it can become removed by splicing of the Ty1 RNA when this is produced in the presence of galactose. If this spliced RNA subsequently becomes reverse transcribed and integrated by the Ty1 integrase into the yeast genome, the now intron-less yeast *HIS3* gene can now be expressed, and the resulting yeast strain is converted to a *HIS*⁺ phenotype. Hence, this assay system allows the efficiency of retrotransposition to be determined by quantifying the ability of the yeast strain being tested to generate *HIS*⁺ colonies after galactose induction.

As shown in Fig. 1, a yeast strain deficient in the gene for yeast Ku70 (*YKU70*) gives rates of retrotransposition that are 5 to 10-fold lower than

that of the control yeast strain. That this defect is due to inactivation of *YKU70* is revealed by the fact that the defect is observed with several independently-derived *yku70* mutant strains (data not shown). Moreover, the transposition defect is fully complemented when the *yku70* mutant strain contains an episomal plasmid containing the *YKU70* gene. This demonstrates that Yku70p plays an important role in Ty retrotransposition.

Yku80p is also required for effective transposition by Ty1 but components of other DNA repair pathways tested are not

To see whether the transposition defect observed above is specific to mutations in *YKU70*, yeasts defective in other DNA repair components were tested for Ty1 transposition. As shown in Fig. 2, inactivation of *YKU80*, the gene for yeast Ku80, also leads to a dramatic decrease in retrotransposition frequencies. By contrast, inactivation of the DNA repair gene *RAD52* or the gene for the putative DNA repair factor Sgs1p do not have a significant effect on the frequency of retrotransposition. It is therefore concluded that Ty1 retrotransposition is affected negatively by mutations in the genes for Yku70p and Yku80p but not by mutations in other DNA repair pathways analysed.

Ku is required for efficient transposition by other types of retrotransposon

To see whether the effect of Ku on retrotransposition is specific to Ty1, or whether it extends to other retrotransposable elements, transposition of the divergent retrotransposon Ty5 was also assessed. Strikingly, the efficiency of Ty5 transposition is reduced over 80-fold when yeast strains possess mutations in either *YKU70* or *YKU80* (Fig. 3). By contrast, inactivation of the DNA repair gene *RAD52* has no significant effect on Ty5 transposition rates. Other studies indicate that mutations in *YKU70* or *YKU80* also impair transposition by the yeast retrotransposon Ty3 (data not shown). Taken together, these data reveal that defects in Ku lead to dramatically reduced transposition frequencies of three divergent retrotransposons. This therefore reveals that Ku affects a fundamental aspect of the retrotransposon life cycle.

20

Yeast Ku does not affect the formation of functional virus-like particles

There are several stages in the Ty life cycle at which Ku could function. First, Ku could affect the synthesis of the virus-like particles (VLPs) by affecting the synthesis of the Ty RNA, the synthesis of other viral components, or the reverse transcription of the RNA and its assembly into an infective VLP.

25

Second, Ku could affect the stability of the VLP or the cDNA that it contains. Third, Ku could affect the ability of the VLP to integrate into target DNA.

Fourth, Ku could be involved in DNA repair steps that occur subsequent to the initial integrase-mediated DNA strand cleavage and transfer reactions.

To begin to address which step(s) is affected by Ku, Tyl VLPs that had been produced in Ku positive and Ku negative yeast strains were purified and analysed. As shown in Fig. 4, through assaying for VLP-associated reverse transcriptase activity, similar amounts of VLPs are generated in the absence of Ku as in its presence. Furthermore, analyses revealed no consistent differences in the polypeptide compositions of VLP preparations derived from Ku positive and Ku negative yeast strains (data not shown). In addition, analysis of the two VLP preparations did not reveal qualitative or quantitative differences in the viral cDNA (data not shown).

To see whether the VLPs isolated from Ku positive and Ku negative yeast cells differ functionally, the abilities of the two VLP preparations to integrate into a plasmid DNA molecule *in vitro* were compared. As shown in Fig. 5, VLPs isolated from Ku deficient cells are no less active in mediating cDNA integration than the VLPs isolated from Ku positive yeasts.

Taken together, these data suggest that Ku does not affect the synthesis, stability or inherent

catalytic activity of Ty element VLPs.

*Identifying the step of the retrotransposon/retrovirus
life cycle that is affected by the Ku-associated DNA
repair apparatus*

There are several remaining possibilities for how
Ku potentiates retrotransposition and all are testable.

(1) It is possible that Ku binds to the
retrotransposon or retrovirus cDNA in the VLPs or the
retrovirus particle (RP) as a way to increase particle
stability or rate of assembly. This possibility may be
tested by using antibodies against Ku subunits in
immunoprecipitation and western blotting studies with
VLPs and virus particles.

(2) Ku could protect the ends of the cDNA from
nucleases or other DNA modifying enzymes. This could
occur when the cDNA is in the VLP or RP and may be
tested simply by determining the precise sequence of
the Ty cDNA ends by cloning and sequencing of the cDNA
ends derived from VLPs or RPs, or by ribonuclease or S1
nuclease mapping procedures (Ausubel et al., 1991).

(3) Ku may prevent "auto-integration" processes
leading to a destruction of the VLP or RP cDNA by one
molecule integrating into another. Auto-integration
events have been demonstrated previously (Lee and
Craigie, 1994 and references therein), so it will be
easily possible to test whether Ku is able to affect
this process. Such assays will utilise VLPs or

infective RPs in *in vitro* transposition reactions in the presence or absence of purified yeast Ku (Feldmann and Winnacker, 1993) or human Ku (Dvir et al., 1993; Hartley et al., 1995).

5 (4) Ku could function *in vivo* to tether the integration machinery to sites on the chromosomal DNA (this type of process has been shown to affect the efficiency of transposition; Bushman, 1994). To test this, the ability of Ku to interact with VLPs or RPs
10 can be assessed *in vitro* by standard biochemical assays or by protein affinity chromatography procedures (eg. tether Ku to an insoluble support and see whether this retains VLPs or Rps).

 (5) Ku could affect the efficiency of integration
15 or could drive the reaction to completion by preventing reverse reactions from taking place (reversal of integration can take place *in vitro*; for example, Chow et al. 1992). This may be tested by assessing the effect of adding purified yeast or mammalian Ku to *in*
20 *vitro* retrotransposition reactions using purified integrase proteins or with VLPs or RPs (for example, Brown et al. 1987; Eichinger and Boeke, 1988; Fujiwara and Mizuuchi 1988; Fujiwara and Craigie 1989; Craigie et al. 1990; Bushman et al., 1990; Katz, et al., 1990;
25 Bushman and Craigie 1991; Pryciak et al. 1992; Moore and Garfinkel 1994; and references therein). In addition, effects of Ku on integration reactions may be investigated by assaying the effects of extracts from

Ku-containing or Ku-deficient mammalian or yeast cells to *in vitro* integration assays.

(6) Ku could be involved in the repair of the integrated products - integrase is capable of joining the viral cDNA to another DNA molecule but these products retain nicks and/or gaps and these must be processed before the DNA can be propagated (for example, Bushman et al., 1990; Craigie et al., 1990). It is possible that the Ku-associated DNA repair apparatus performs these functions. Such models may be tested by introducing the nick-containing intermediates of *in vitro* retrotransposon/retrovirus integration reactions into Ku positive or Ku negative yeast or human cells, then analysing their repair *in vivo*. This type of analysis may also be conducted with artificial substrates that mimic nicked integrase-generated retrovirus/retrotransposon integration intermediates. Another way to address this is to analyse the processing of natural or artificial nicked substrates *in vitro* using extracts of yeast or human cells that are proficient or deficient in components of the Ku-associated DNA repair system, or by using (part) purified components of the Ku-associated DNA repair system.

It is important to note that for performance of the present invention in various aspects it is not necessary to know how Ku is involved in the retrovirus/retrotransposon life cycle. It can simply

be noted that it is so involved and that, as demonstrated herein, disruption of Ku function has an anti-retroviral/anti-retrotransposon effect.

5 *Inhibition of yeast Ku activity leads to reduced Ty transposition frequencies*

 The loss of yeast Ku function affects Ty transposition rates (established above) provides indication that, Ty transposition frequencies will be
10 reduced in Ku positive cells by inhibitors of Ku action.

 One approach to demonstrate this principle involves introduction into YKU70/YKU80 strains of plasmid molecules that direct the expression of mutated
15 derivatives of Yku70p or Yku80p (using the vectors such as those described previously; Boulton and Jackson, 1996; Boulton and Jackson, 1996). The Ku mutants studied may for example include N-terminal deletion mutants, C-terminal deletion mutants, and point mutants
20 in regions of the protein that are conserved throughout evolution (eg. Feldmann and Winnacker, 1993; Boulton and Jackson, 1996). Since the subunits of Ku function as a heterodimer and work in association with various other proteins, various mutated Ku molecules able to
25 interact with their partner or with other components of the system will form non-functional complexes. Such dominant negative mutations, when used in Ty transposition studies, provide indication that

inhibition of Ku function can impair Ty retrotransposition.

Definition of the regions of Yku70p and Yku80p that function in Ty integration

5 The regions of the two Ku subunits that function in Ty retrotransposition may be defined by assessing the abilities of mutated derivatives of YKU70 and YKU80 to complement the transposition deficiencies of yku70
10 and yku80 mutant strains, respectively (using vectors such as those described previously; Boulton and Jackson, 1996; Boulton and Jackson, 1996). The ability of the mutated Yku70p and Yku80p derivatives to complement the DNA repair, radiosensitivity and
15 telomeric loss phenotypes of Ku-deficient yeasts may also be tested using standard procedures (Boulton and Jackson, 1996; Boulton and Jackson, 1996; Porter et al., 1996). Similar approaches may also be used to define important functional regions of mammalian Ku70
20 and Ku80 and define functional regions of other components of the Ku-associated DNA repair apparatus (see below).

 The identification of regions that affect retrotransposition selectively, may lead to the
25 development of drugs that interfere with retrotransposition but do not affect other Ku-dependent processes.

Effects of other components of the Ku-associated DNA repair apparatus on retrotransposition.

The results obtained with Ku, lead to an expectation that other proteins that function in Ku-associated processes will play a role in retrotransposon and retroviral integration. Recent unpublished data from the inventor's laboratory indicate that the products of the yeast genes *RAD50*, *XRS2*, and *MRE11* function with Yku70p and Yku80p in DNA DSB rejoining (S. Boulton, J. Downs, and S.P. Jackson, unpublished data). DSB rejoining (S. Boulton, J. Downs and S.P. Jackson, unpublished data). Thus, disruption of the *MRE11*, *XRS2*, or *RAD50* genes results in defects in illegitimate end-joining as ascertained by plasmid repair assays and in radiosensitivity experiments such as those described previously (Boulton and Jackson, 1996a, 1996b). These defects are the same as those observed in strains disrupted for *YKU70* or *YKU80*. (Boulton and Jackson, 1996a and 1996b). Furthermore, no additional defect in DNA repair is observed in double-mutant strains deficient in Ku plus any one of these other genes (*MRE11*, *XRS2*, *RAD50*). These data therefore indicate that *MRE11*, *XRS2* and *RAD50* function in the same DNA DSB repair pathway as Ku. Further unpublished results show that the yeast gene that we term *LIG4* is also part of the Ku-associated DNA repair apparatus. This corresponds to open reading frame YOR005c on the right arm of *S. cerevisiae* chromosome

XV, accession number Z74913 of the yeast genome database, and encodes a protein with strong sequence similarity to mammalian ligase IV (Wei et al., 1995). Disruption of *LIG4* results in defects in illegitimate end-joining as ascertained by plasmid repair assays and in radiosensitivity experiments and these defects are the same as those observed in strains disrupted for *YKU80* (Boulton and Jackson, 1996a, 1996b). Furthermore, no additional defect in DNA repair is observed in double-mutant strains deficient in Ku plus *LIG4*, indicating that these genes function in the same pathway. The effect of mutating these genes on Ty retrotransposition frequencies may be tested using assays similar to those described for Ku.

Should a yeast homologue of the mammalian XRCC4 protein (Li et al., 1995) be identified, this will also be expected to be involved in Ty transposition because the mammalian factor functions as part of the Ku-associated DNA repair apparatus (and see below) and defects in mammalian XRCC4 negatively affect retrovirus integration (see below). If any other components of the yeast Ku-associated DNA repair apparatus are defined, these will also be tested for their role in retrotransposition. Should mutation of any of these genes lead to reduced retrotransposition, then these factors, and their homologues in other organisms, will be attractive targets for the development of novel anti-retroviral agents.

Steps to determine the site(s) of involvement of additional factors in retrotransposition and the development of strategies for drug screening etc. will be analogous to those described above and below for the Ku subunits.

Defects in the Ku-associated DNA repair system lead to reduced infectivity by retroviruses in mammalian cells

To see whether Ku affects retrotransposon and retrovirus propagation in mammalian systems, the abilities of a mammalian retrovirus to integrate into the genome of mammalian cells that are proficient or deficient in the Ku-associated DNA repair apparatus were analysed. For these studies, a derivative of the Moloney murine leukaemia virus (Mo-MLV) was used to infect various rodent cell lines (to allow infection, the virus was "pseudotyped" with the vesicular stomatitis virus G protein; Burns et al., 1993; Hopkins, 1993; Yee et al., 1994; and references therein).

Notably, the generation of retroviral integrations is reduced several-fold in the Ku80-deficient *xrs-6* cell line compared to its parent, K1. In addition, it was found that the virus integrates with lower efficiency into the XRCC4-deficient cell line XR-1 than it does into K1 cells.

These data therefore reveal that components of the mammalian Ku-associated DNA repair apparatus play

important roles in the generation of stable integration products by mammalian retroviruses. Given that this is also the case in yeast, it can be reasonably concluded that the requirement for the Ku-associated DNA repair apparatus in efficient retroviral and retrotransposon integration is ubiquitous throughout the eukaryotic kingdom.

In light of the above data, the involvement of other components of the mammalian Ku-associated DNA repair apparatus in efficient retroviral and retrotransposon integration may be established by assessing the efficiency of retroviral integration into cell lines deficient in other components of this system. For example, mouse, hamster and human cell lines exist that are deficient in the catalytic subunit of DNA-dependent protein kinase (DNA-PKcs), which is part of the Ku-associated DNA repair machinery in mammalian cells (see e.g. Blunt et al., 1995; Lees-Miller et al., 1995; for reviews, see Jackson and Jeggo, 1995; Jackson, 1996).

Where naturally occurring mammalian mutants do not already exist, targeted disruption of loci by standard "gene knock-out" approaches may be used to derive the desired cell type (eg. defective in mammalian ligase IV, or defective in homologues of yeast Rad50p, Mre11p and Xrs2p). Alternatively, studies may be performed in cells rendered deficient in repair due to the expression of dominant negative mutants of factors such

as ligase IV, Rad50p, Mrellp, and Xrs2p. Since mammalian ligase III is very like ligase IV in sequence, cells deficient in ligase III or the associated XRCC1 protein may be tested for their ability to mediate retroviral or retrotransposon integration (the Chinese hamster ovary cell line derivatives EM-9 and EM-C11 lack functional XRCC1; Thompson et al. 1990; Caldecott et al. 1996; and references therein).

By using a variety of mammalian retrovirus types, it will also be straightforward to show that, as in yeast, the mammalian Ku-associated DNA repair apparatus plays an important role in the integration of a wide range of retrovirus into mammalian cells. Test viruses include other animal tumour retroviruses, human T-cell leukaemia viruses and HIV-1, which causes AIDS (HIV can be used to infect human cells or can be derivatised to infect rodent cells; eg. Naldini et al., 1996).

As described above for the yeast system, *in vivo* and *in vitro* studies may be employed to ascertain precisely at which step(s) the Ku-associated DNA repair machinery functions in mammalian retroviral and retrotransposon integration. Furthermore, already available assays may be used to define the roles performed by individual components of the machinery and define the precise regions of the various polypeptides that are critical for function. This should facilitate the design of assays for substances that disrupt this

function and so are indicated for use as anti-retroviral agents.

*Inhibitors of the Ku-associated DNA repair apparatus .
protect against retroviral infection.*

5 As described above for the yeast system, dominant negative derivatives of Ku subunits and other components of the Ku-associated DNA repair apparatus can be used to show that inhibition of this repair
10 apparatus in normal cells results in decreased ability to stably integrate retrovirus into the host cell genome. For these studies, stable mammalian cell lines are generated that express the dominant negative mutant proteins. Wild-type mammalian cell lines are also
15 treated with various drugs to show that this results in inhibition of retroviral integration.

 In this regard, the compounds wortmannin and LY294002 have been shown to inhibit Ku-associated DNA-dependent protein kinase (DNA-PK) activity (Hartley
20 et al., 1995; Vlahos et al. 1994; also unpublished data from the inventor's laboratory). Other DNA-PK inhibitors that are identified may be tested for effects. Although it is most simple to test agents initially on tissue culture cells, preliminary studies
25 may subsequently be extended to involve animals such as mice (mice deficient or proficient in components of the Ku-associated DNA repair apparatus will be employed) and, ultimately, to human subjects.

Development of assay procedures for agents that reduce retroviral infectivity by disrupting the Ku-associated DNA DSB repair apparatus.

Determination of the mechanism by which the Ku-associated DNA repair machinery functions in retrovirus and retrotransposon integration facilitates design of assays that recapitulate these functions in defined *in vivo* or *in vitro* assays. Such assays are among those that may be used in screens for inhibitors, identifying substances as potential anti-retroviral agents.

Assays include those that measure full retroviral integration or just measure the step(s) of the process that are affected by the Ku-associated machinery. Although *in vitro* assays may be the easiest to employ in screens, yeast or mammalian cell lines may provide useful *in vivo* systems to test for effects. In this regard, human Ku70 and Ku80 may be expressed in yeast cells for assessment of Ty transposition or other aspects of Ku-dependent events. Such a "humanised" yeast system may be used to screen for molecules that interfere with human Ku function.

Another screen that may be used to identify novel anti-retroviral agents is one that identifies inhibitors of Ku-associated DNA-PK activity (for example, Dvir et al., 1992; Gottlieb and Jackson, 1993; Finnie et al., 1995; and references therein).

Other screens may be for compounds that inhibit

enzymatic activities associated with ligases III or IV, Rad50p and Mre11p (both putative nucleases), interfere with protein-protein interactions within the Ku-associated repair apparatus (for example, between Ku and other components of the apparatus, between Rad50p, Mre11p and Xrs2p, between XRCC1 and ligase III, or between XRCC4 and ligase IV), or interfere with associations between the repair machinery and the DNA substrate. Peptides or peptide mimetics that resemble important regions of components of the Ku-associated repair system may be tested in addition to small molecule drugs.

Once identified through such routes, drugs (and their derivatives) may be tested in cell, animal and ultimately human systems for effects on retroviral and retrotransposon integration and on retroviral infection.

Other applications for activators or inhibitors of the Ku-associated DNA repair apparatus

In addition to being of use in the identification and design of anti-retroviral drugs for use in vertebrate systems, activators or inhibitors of Ku-associated processes will also be of utility in other systems.

For example, retrotransposons are very abundant in the genomes of many higher plants and many transpose relatively frequently (Wessler, 1996). Indeed, much of

the problematic somaclonal variation that occurs after plants are regenerated from protoplast tissue cultures appears to arise through retrotransposition events (Wessler, 1996). Aspects of the present invention may
 5 be used for suppressing the generation of somaclonal variation in plants by inhibition of the plant Ku-associated DNA repair apparatus.

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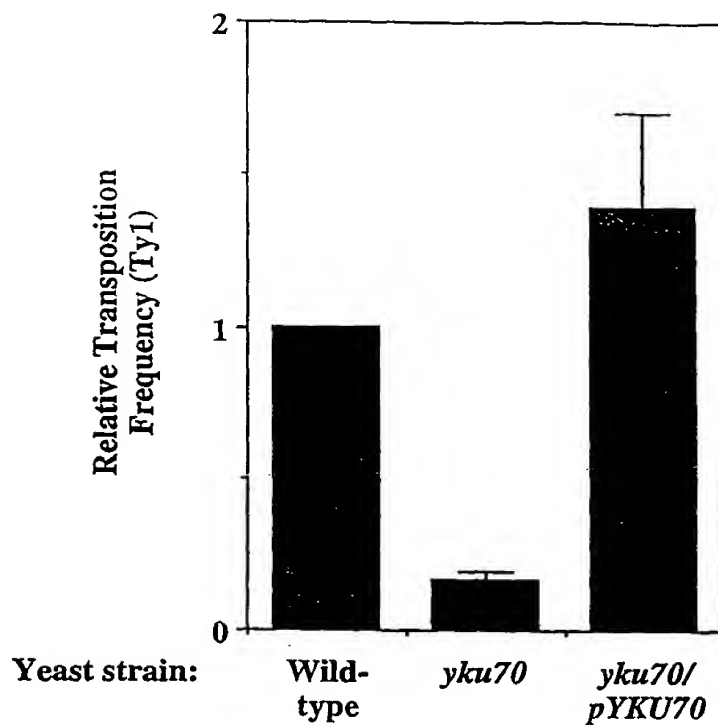


Figure 1

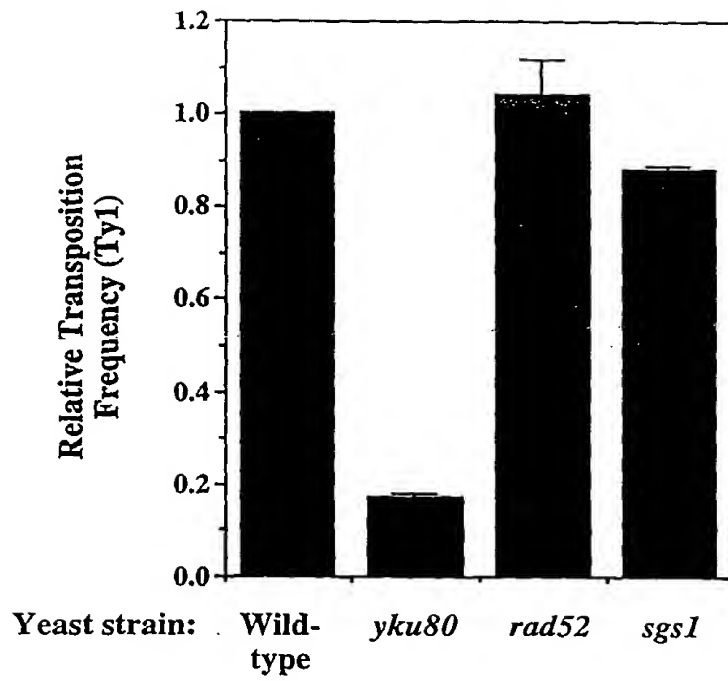


Figure 2

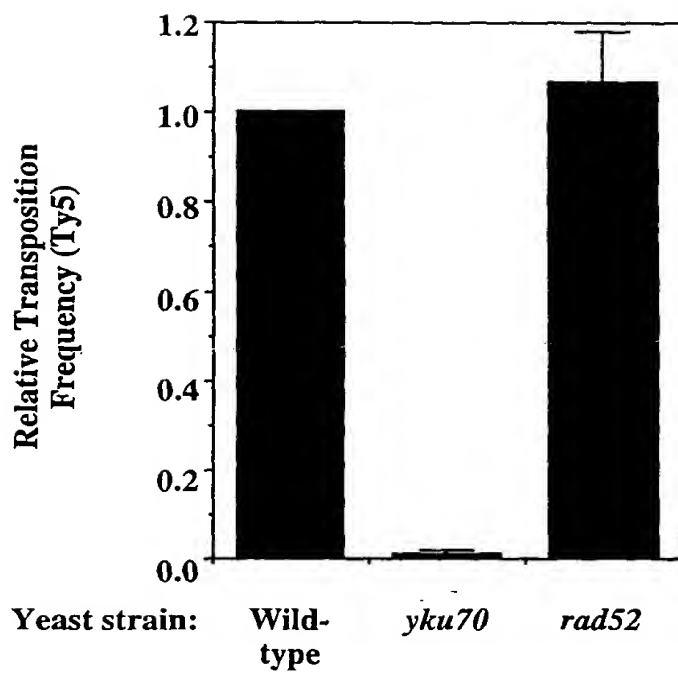


Figure 3

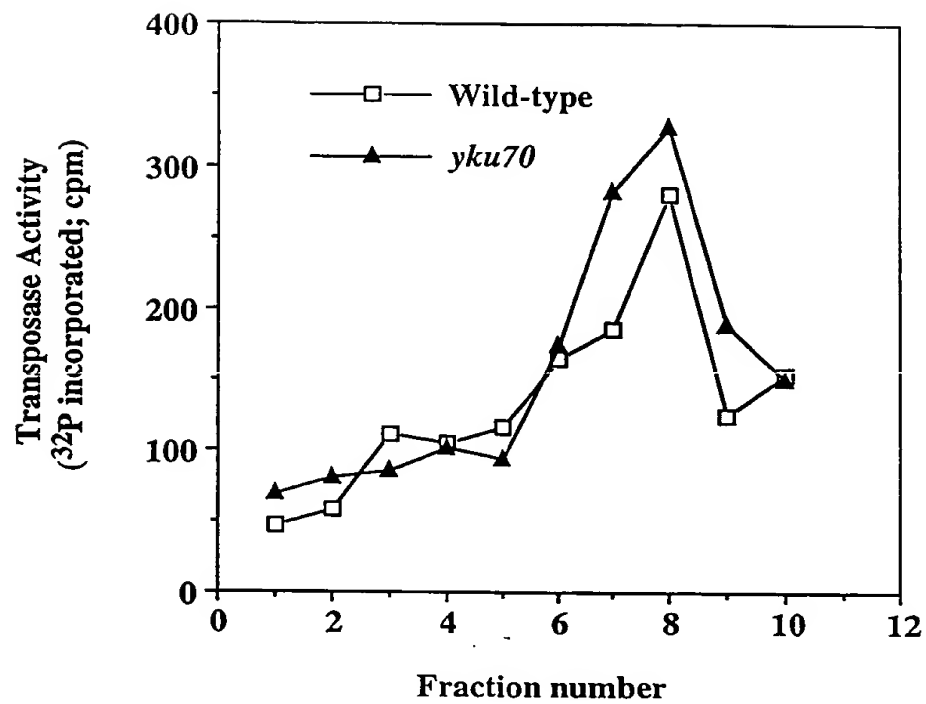


Figure 4



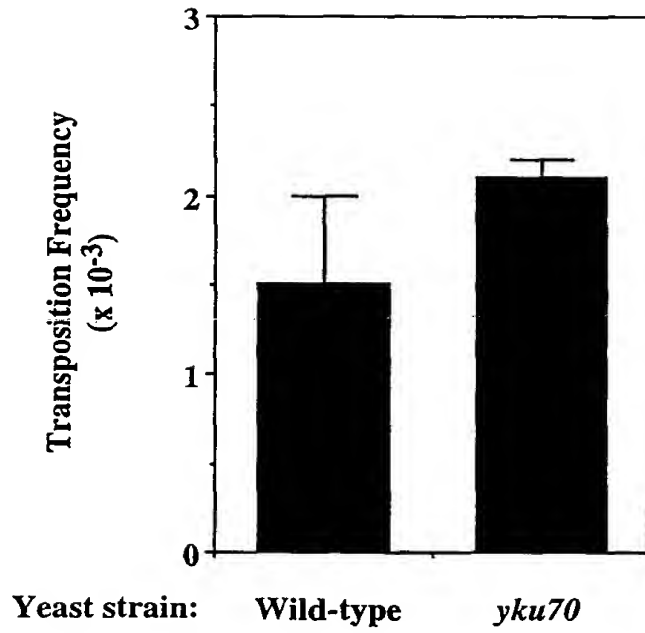


Figure 5

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